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14. ABSTRACT

The subject of these studies is the development of targeted vector for selective infection and killing of prostate cancer cells. The resultant vector is to be administered to prostate cancer patients to find, infect and destroy tumor cells. Targeting of the vector to prostate tumors is to be accomplished via incorporation into its capsid of ligands, which selectively bind to prostate-specific membrane antigen (PSMA). Research accomplishments: (i) two targets, recombinant sPSMA protein and 293/PSMA cells, have been developed for identification of PSMA-specific peptides; (ii) a panel of monoclonal anti-PSMA antibodies has been derived; (iii) a single chain (scFv) version of one of these mAbs has been incorporated into a filamentous phage; (iv) an ELISA-based screening procedure has been established; (v) two scFv-targeted phage vectors each expressing either GFP or HSV tk.

15. SUBJECT TERMS
prostate cancer, prostate-specific membrane antigen

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INTRODUCTION

The subject of this study was the development of a viral vector capable of selectively infecting and killing prostate cancer cells. The intended use of such a vector was for gene therapy of prostate cancer patients, whereby the vector administered to patients would find, infect and destroy tumor cells. This work was to employ the concept of genetic targeting of vector to tumor-specific cell surface molecules. Targeting of the vector to prostate tumor cells was to be accomplished via genetic incorporation into its capsid of a ligand, which selectively binds to a major marker of prostate tumors, the prostate-specific membrane antigen (PSMA). The selectivity of the designed vector for PSMA-positive cells and the efficacy of the cell killing was to be assessed *in vitro*.

REPORT BODY

Specific Aims. The originally stated ultimate goal of this study, the generation of highly specific conditionally-replicative adenovirus vectors (CRAds) for treatment of prostate cancer (PCA), was to be achieved via realization of the following specific aims.

Specific Aim 1. To identify PSMA-specific peptide ligands by means of phage display technology.

Specific Aim 2. To derive recombinant Ad5 vectors targeted to PCA cells via genetic incorporation PSMA-specific peptides in the Ad fiber protein.

Specific Aim 3. To generate CRAd derivatives of PSMA-targeted vectors and demonstrate their oncolytic advantages over parental CRAd vector *in vitro*.

Experimental work and results.

Specific Aim 1. The work began with the expression and purification of recombinant form of soluble PSMA protein, sPSMA, to be used later in the project as a target for phage library biopanning. sPSMA is a genetic fusion of the extracellular domain of human PSMA with the Fc domain of human immunoglobulin. Fc was being used in this construct for two purposes: first it facilitates the expression and secretion of sPSMA fusion; second, it was exploited as an affinity tag during the purification of the protein from the culture medium.

To this end, we have employed the previously designed replication incompetent Ad vector, Ad5.HuFcPSMA (1), encoding sPSMA to direct its expression in eukaryotic cells. The protein was produced and secreted by the A549 human lung carcinoma cells infected with Ad5.HuFcPSMA. Purification of the protein from culture medium was done using HiTrap™ Protein A HP affinity columns from Amersham Biosciences. This resulted preparation of sPSMA, which was over 95% pure according to SDS-PAGE analysis (Fig. 1). Additional purification was achieved by gel filtration chromatography on Superdex 200HR column (Amersham Biosciences). The final yield of sPSMA protein was 12mg.

In order to have an alternative target for phage library biopanning, we also generated a cell line stably expressing full size, membrane-bound human PSMA. To this end, we designed a plasmid vector encoding PSMA by cloning its cDNA into pcDNA3 expression vector (Invitrogen). The resultant plasmid was used for transfection of 293

cells with subsequent selection of clones in the medium containing G418 antibiotic. 293 cells were chosen because their PSMA-expressive derivative could serve two distinct and important roles in this project: in addition to being a cell target for phage library biopanning, it could also be used subsequently for the rescue and propagation of the proposed conditionally replicative, PSMA-targeted Ad vector. A panel of G418-resistant clones was then tested for PSMA production by Western blot employing Y-PSMA1 anti-PSMA monoclonal antibody (mAb) (Yes Biotechnologies), which recognizes denatured form of the protein. This allowed for the identification of several 293-derived cell lines, 293/PSMA, expressing PSMA at high levels (Fig. 1B).

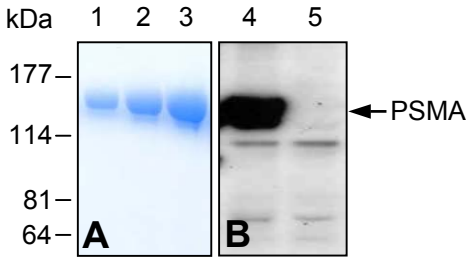


Figure 1. Expression of the soluble and membrane bound forms of human PSMA. A. SDS-PAGE of purified sPSMA protein. Lane 1, sPSMA (1μg), lane 2, sPSMA (2μg), lane 3, sPSMA (4μg). B. Expression of PSMA in 293 derived 293-PSMA cells (clone #22). Western blot was done with aliquots of cell lysates corresponding to 5 μg of total soluble cell protein and probed with Y-PSMA1 anti-PSMA Ab. Lane 4, 293/PSMA cells, lane 5, 293 cells.

As 293 cells normally do not produce PSMA, it was important at this juncture to confirm that PSMA expressed in 293/PSMA cells retains its native configuration and is exposed at the cell surface. While the method best suited for this purpose would be flow cytometry employing anti-PSMA Ab, the only Ab we had (Y-PSMA1 from Yes Biotechnologies) did not bind native PSMA and thus could not be used for this assay. Therefore, in collaboration with the UAB Hybridoma Core Facility we have generated a panel of mAbs possessing the desired specificity. Briefly, sPSMA protein expressed in A549 cells (see above) was used for immunization of mice, splenocytes isolated from the animals were fused with myeloma cells, and the production of anti-PSMA Ab by the resultant clones was tested by ELISA employing sPSMA. Positive samples were then tested by flow cytometry for binding to LNCaP human prostate cancer cells known to be PSMA-positive (Fig. 2). “Counter selection” was done in the format of Western blot using fully denatured sPSMA (data not shown). In the aggregate, these two assays identified the hybridoma clones producing the desired anti-PSMA mAb, which only bind native form of the protein. These mAbs were subsequently used in flow cytometry setting to confirm the cell surface localization and correct folding of PSMA protein produced in 293/PSMA cells (Fig. 3).

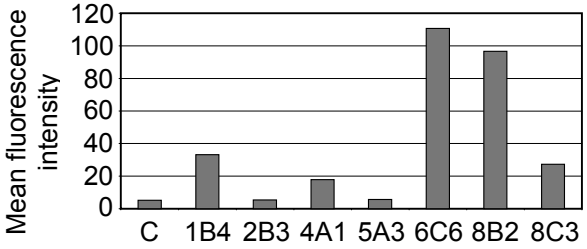


Figure 2. FACS analysis of anti-PSMA mAbs using LNCaP cells. $5x10^5$ LNCaP cells were incubated first with selected anti-PSMA Abs (clone #s are shown at the bottom of the graph), and then with secondary anti-mouse FITC-conjugated Ab. C, negative control (no primary antibody added).

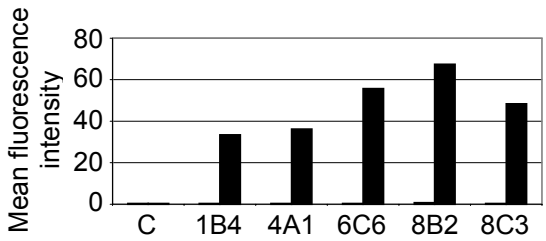
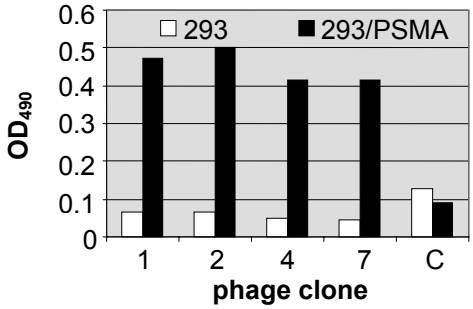


Figure 3. Confirmation of PSMA expression on the surface of 293/PSMA cells by FACS analysis. $5x10^5$ 293 (white bars) or 293/PSMA (black bars) cells were incubated first with selected anti-PSMA Ab (shown below the graph), and then with secondary anti-mouse FITC-conjugated Ab. C, negative control (no primary antibody added).

Another important development in the project is the generation of a filamentous phage displaying on its particle an anti-PSMA single chain antibody (scFv). This phage capable of selective binding to PSMA has been designed to be used as a positive control in FACS and ELISA assays later in the project, when peptide-bearing phage species isolated from the phage display libraries were supposed to be tested on sPSMA or 293/PSMA cells. To this end, total RNA isolated from one of hybridomas cell lines, 6C6 (see above), was used as a template for RT-PCR to amplify the sequences encoding individual V_L and V_H domains of the antibody. These sequences were then sequentially incorporated into the pSEX81 phagemid vector to assemble the entire open reading frame of the scFv. The phagemids were then converted into phages by infection of bacteria with helper phage. The resultant phage mini-library was then panned against recombinant sPSMA protein and individual clones were randomly selected from the enriched pool for ELISA screening on PSMA protein. Additionally, these phage clones were used for ELISA on fixed 293/PSMA cells, which employs the conditions of the cells fixation that do not affect the native structure of PSMA. As seen on Fig. 4, the 6C6-derived scFv exhibited specific binding to native, cell membrane-anchored PSMA.

Figure 4. Confirmation of binding specificity of the 6C6-derived scFv. Four scFv-displaying phage clones that showed binding to recombinant sPSMA protein have been tested in an ESLIA on fixed cells. These phages were amplified, their titers determined and 2×10^9 infectious particles of each phage were used for binding to either 293 or 293/PSMA cells seeded in 96 well plate. The clones have been designated 1, 2, 4, and 7. C, a control phage clone, which showed no binding to sPSMA protein.



Therefore, we have prepared two targets, the protein (sPSMA) and cells (293/PSMA), suitable for identification of PSMA-specific ligands later in the project. Furthermore, we have derived a “positive control” phage clone capable of binding PSMA, which could be used for screening the phage clones isolated by library biopanning.

To identify PSMA-specific peptide ligands we used a number of approaches which differed from each other by (i) the target used (sPSMA or 293/PSMA) cells; (ii) type of phage display library. The two libraries in our possession were the Ph.D-7™ and Ph.D-12™ (New England Biolabs) that differed by the size of the random peptide sequence displayed of the particle (7 and 12 aa, respectively). First, we started the work with the Ph.D-12™ library. However, our experience with this library was rather negative, as it turned out to contain a very significant percentage of the wild type, insertless phage, which gradually overgrew the recombinants in the screened population and made the identification of true binders impossible as early as at the fourth round of biopanning. As judged by the ratio of phage eluted from the substrate to that originally added to biopanning reaction, at this early point in the selection we have not achieved the enrichment of the PSMA-binding phage species that would be sufficient to identify and isolate them from the pre-selected pool. This occurred regardless of the target used in the selection. We have made a number of attempts to sequence an insert-encoding region of the genomes of individual phage species (non-contaminants) after four rounds

of selection. Over two hundred phage clones have been characterized in this manner and several sequences found in the population more than once have been identified. However, when tested individually on 293/PSMA and 293 cells in ELISA, all these phage species showed no binding to PSMA.

Next, we used the Ph.D-7^{TM} library for panning on 293/PSMA cells employing the method described by Rasmussen *et al.* (2) with modifications suggested by Dr. Tatyana Samoylova (Auburn University). In brief, an aliquot of Ph.D-7^{TM} library corresponding to $2x10^{10}$ plaque forming units was first incubated in suspension with 293 cells to counter-select against those phage species, which bound to the surface of these cells. Unbound phage were collected and panned against the 293/PSMA cells. After extensive washing of the cells to remove unbound phage, the phage bound to cells was eluted with acid buffer and after pH neutralization was saved for subsequent amplification. In order to rescue the phage, which could have been internalized by the cells, after acid elution we lyzed the cells and saved the recovered phage as a separate pool. Both samples of the phage were then amplified in *E.coli* and used in subsequent rounds of biopanning. Of note, the phage pools eluted from the cell surface and that recovered from the lyzed cells were used in subsequent round of selection separately from each other. In each round of selection using “eluted” phage, only phage eluted from the cells was collected; similarly, in the selection procedure employing phage initially recovered from the lyzed cells only internalized phage was recovered and re-amplified. In contrast to our experience with Ph.D-12^{TM} library, this time we have not seen any contamination of the library by the wild type phage, which has allowed us to reach the sixth round of selection. As a result, the ratio of phage eluted from cells to that added to the cells for binding has changed from $2x10^{-7}$ to $4.3x10^{-5}$, suggesting that some enrichment of PSMA-binding species has taken place. At this point, we have randomly picked 92 phage clones and partially sequenced their genomes to determine the sequences of the displayed peptides. Alignment of the sequences of the peptide inserts showed the following results. All clones (a total of 48) recovered from the cell lysates contained the same peptide *ALPQWLL*. A nearly identical peptide sequence *ALPSWLL* was found in 30 out of 44 phage clones eluted from the surface of 293/PSMA cells. Another seven clones in eluted phage pool displayed two highly homologous peptides *KLWVIPQ* (6 clones) and *KLWSIPR* (1 clone). Another two motifs found in the “eluted” phage pool, *SAVHLSA* (4 clones) and *HTVGASS* (3 clones) did not have any significant homology between themselves or to the other identified motifs (above). The high degree of homology between the groups of these peptides suggested that we may have succeeded in the identification of the true binders.

However, contrary to our expectations, in our subsequent studies we have not been able to prove that the peptide ligands mentioned above indeed bind PSMA protein. Specifically, when individual phage species representing the aforementioned peptides were tested for binding to the PSMA-expressing cells, none of them demonstrated any binding to these cells above background level. Based on these finding we chose to exclude these motifs from the study.

As the project could not move on without targeting ligands being identified, we have explored another approach. This time, instead of relying on the libraries available to us

from commercial vendors, we chose to switch to libraries and biopanning methods developed by others. To this end, we have established two key collaborations with scientists whose expertise in the field of biopanning was significantly better than ours. In particular, we worked on this problem together with our Consultant, Dr. Renata Pasqualini (MD Anderson Cancer Center) by soliciting her help in identifying the desired peptides. In this work the phage display libraries designed in Dr Pasqualini's laboratory were panned against our 293/PSMA using the *BRASIL* method recently published by her group (3). Unfortunately, this collaborative effort has ended the same way as our own (above): while some "consensus" peptide motifs were seen in the enriched library pools after several rounds of biopanning, none of those phage species showed any significant binding to the cell-anchored PSMA when tested individually. Similarly, negative results were obtained with these phage clones in ELISA utilizing recombinant Fc-PSMA protein.

In parallel, we have explored yet another avenue to develop PSMA-specific ligands for Ad targeting by using the technology of the so-called "antibody mimics", which is being developed in a number of laboratories worldwide as an alternative to the utilization of traditional Abs (4). The need for such alternative proteins, which would possess the desired binding specificities, is dictated by a number of limitations characteristic of traditional antibodies. Some of these limitations, such as difficulties of production of recombinant Ab, instability of Ab in certain subcellular compartments (cytoplasm and nucleus), *etc*., are direct consequences of a native structure of a typical Ab molecule. For instance, the disulfide bonds, which stabilize the structure of individual domains of and Ab protein, VH and VL, cannot be formed at reducing conditions of the cyto- or nucleoplasm, thereby making the use of Abs in these cellular compartment highly problematic. This problem has direct relevance to our Ad vector targeting work, as it prevents us from using Abs and their derivatives as targeting ligands for Ad: because all the components of Ad capsid follow the biosynthetic pathways of nucleus-localized proteins, they may not serve as substrate for disulfide isomerases (localized in the endoplasmic reticulum). The same holds true with respect to any protein moiety genetically fused to an Ad capsid protein. Recently published studies have provided convincing experimental evidence for these theoretical considerations (5). Antibody mimics are being designed such that while retaining the capacity of efficient and selective binding to a target, they do not contain any disulfide bonds and thus are free from the aforementioned limitations. To our mind, they represent an ideal choice as ligands for Ad targeting. In this regard, we have been fortunate to establish a collaborative arrangement with Dr. Andreas Pluckthun (University of Zurich), who is the Director of one of the world's leading laboratories in Ab and Ab mimics research. This allowed us to get Dr. Pluckthun's help in developing PSMA-specific ligands by using his novel approach employing the so-called designed ankyrin-derived proteins, or DARPins. Toward this end, the sPSMA protein was used for ribosome display-based in vitro biopanning of DARPin-expressing library. Two sequential attempts to select PSMA-binding DARPins were, however, unsuccessful, as no binders were found.

Having completed this part of the project we have, in essence, exhausted all the possibilities to identify the targeting ligands. Because no ligands have been

identified, we could not proceed with making targeted Ad vectors as was originally proposed. The only way to continue the work toward the goal of making a PSMA-specific gene vector was to adapt the 6C6 scFv-displaying phage that was designed earlier in the project. Whereas bacteriophages do not have natural capacity of transducing eukaryotic cells, it has been shown by several groups that some degree of transduction can be achieved by providing the phage with the cell-binding ligand. At this juncture, this rationale was presented to the Sponsor and approval was obtained.

Thus, the genome of this phage vector was further modified to contain a gene cassette that expressed either the humanized version of the green fluorescent protein (hrGFP), or the herpes simplex virus thymidine kinase (HSV tk). While the virions of both vectors were identical and both contained the same targeting moiety, C6C scFv, each of the two vectors was designed to serve a different purpose. Specifically, while the phage encoding the GFP - fuCT/C6C/hrGFP - was designed for the sole purpose of tumor cell imaging, the HSV tk-expressing phage - fuCT/C6C/TK - was developed as a therapeutic vector with a self-imaging capability. In the latter instance, the HSV tk expressed by the phage could be employed either as a prodrug-converting enzyme in the context of the gancyclovir(GCV)-mediated therapy, or as a radiolabeled precursor-converting enzyme thereby facilitating the positron emission tomography(PET)-based imaging.

By providing these phage vectors with reporters we have been able to demonstrate that they could enter a PSMA-positive cell by using PSMA as a surrogate receptor. Most importantly, the gene payload carried by a targeted phage was expressed within the transduced cells allowing for either the visualization of the cell by optical imaging (due to GFP fluorescence) (Fig. 5), or killing those cells in the presence of GCV (Fig. 6).

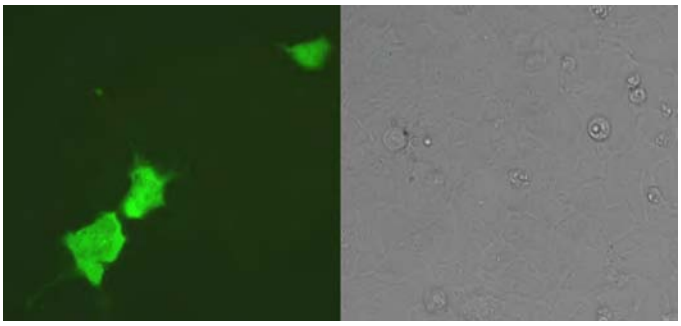


Figure 5. Transduction of PSMA-positive cells with scFv-targeted phage vector. The derivative of the human embryonal kidney cell line 293 that has been designed to stably express human PSMA, 293/PSMA, was transduced by the hrGFP-expressing, PSMA-targeted phage vector fuCT/C6C/hrGFP. Forty-eight hours post-transduction the cells were examined under the fluorescent microscope. Left panel, fluorescent image; right panel, the same field viewed under white light. No fluorecent foci could be seen in a similarly transduced 293 cells that are PSMA-negative.

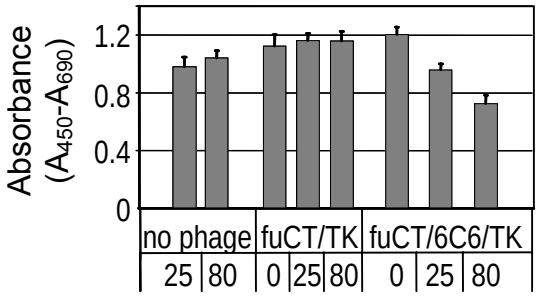


Figure 6. GCV dose-dependent killing of PSMA-positive cells transduced with the HSV tk-expressing, PSMA-targeted phage vector. 293/PSMA cells seeded in a 96-well plate were incubated for 4 h with the fuCT/C6C/TK phage (10^5 phage particles per cell). In thirty-six hours, GCV was added to cells at concentrations of either 25 or 80μM. The cell proliferation rate was assessed using Quick Cell Proliferation Assay Kit (BioVision) five days later.

While the transduction of PSMA-positive cells has been demonstrated, its efficacy was very low even at high infectious dose, which was in line with what other groups have reported for phage vectors. To overcome this deficiency, we chose to take advantage of the recent findings by Dr Pascualini, who discovered that the magnitude and the duration of the transgene expression in tumor cells by the integrin-targeted phage vector could be significantly improved by the incorporation into its genome of the inverted terminal repeats (ITRs) of adeno-associated virus (AAV) (6). With this discovery in mind, in the final phase of the project we redesigned the genome of our PSMA-targeted phage vectors, fuCT/6C6/TK and fuCT/6C6/GFP, by flanking the transgene-containing expression cassette with the ITRs of AAV. The receptor specificity of the new AAV ITR-containing vectors designated fuCT/6C6/TK$_{ITR}$ and fuCT/6C6/GFP$_{ITR}$ was then confirmed by flow-cytometry. This was done on the PSMA-negative 239 cells and PSMA-overexpressing 293/PSMA cells using anti-phage polyclonal antibodies to detect bound phage.

Further, the fuCT/6C6/GFP$_{ITR}$ phage was used to transduce the same cell lines in order to assess the magnitude and duration of the GFP expression. The expression of the transgene was followed by examination of the phage-treated cell cultures under fluorescent microscope. Despite our expectations, no improvements have been seen in either the level of GFP fluorescence, nor the longevity of its expression. Specifically, the proportion of the GFP-positive cells in cultures treated with fuCT/6C6/GFP$_{ITR}$ at 10^5 virions/cell remained below 1%. This fluorescence virtually disappeared within days after the transduction experiment was done. Of note, no substantial increase in the transgene expression was seen even after the multiplicity of transduction was elevated to 10^6 phage particles per cell. Because this level of gene transfer by fuCT/6C6/GFP$_{ITR}$ was just as low as that of the fuCT/6C6/GFP vector (no AAV ITRs), it provided no reason to expect any improvements in the cell killing capacity for the similarly designed HSV TK-encoding fuCT/6C6/TK$_{ITR}$. With the transduction rate being that low, even the so-called “bystander effect”, which has been described for HSV TK/gancyclovir therapy treatment, would not yield any noticeable cell killing. On this basis, the cell killing experiments were ended.

KEY RESEARCH ACCOMPLISHMENTS

- Two different targets, recombinant sPSMA protein and 293/PSMA cells, have been developed and made ready for identification of PSMA-specific peptides
- A panel of monoclonal anti-PSMA antibodies has been derived
- A single chain (scFv) version of one of these mAbs has been incorporated into a filamentous phage to serve as a positive control during characterization of PSMA binding by phage clones isolated from peptide display library
- An ELISA-based screening procedure has been established which employs 293/PSMA cells and allows for the high throughput screening of phages identified by panning
- Two scFv-targeted phage vectors each expressing either GFP or HSV tk.

REPORTABLE OUTCOMES

None.

CONCLUSIONS AND FUTURE DIRECTIONS OF THE WORK

Our findings have shown clearly that the concept of using a bacteriophage vector for targeted gene delivery to and therapy of the PSMA-expressing tumors is unusable. Based on our findings, we conclude that while targeting of a phage vector to PSMA-positive cells is feasible and results in an efficient and specific ***binding*** of the phage particles to these cell targets, the efficiency of the ***gene delivery*** by such a vector is negligible and impractical for the therapeutic use. It is in contrast to the reported successful transduction of tumor cells by the RGD-modified phage vector targeted to integrins (6). This apparent discrepancy may be explained by the fact that in contrast to the well-documented role of integrins in the internalization of viral vectors, no reports exist to prove the involvement of PSMA in the internalization of such agents. Having seen efficient binding of our phages to PSMA and virtually no gene expression, we conclude that binding to PSMA either does not lead to the phage internalization at all, or results in its entrapment in the lysosomes, where the phage particles are degraded, thereby yielding no gene transfer to the nucleus. Also, because no augmentation in gene transfer has been seen after the escalation of the multiplicity of transduction from 10^5 to 10^6 phage particles per cell, we further conclude that the cell entry pathway that results in this low transduction is very limited in its capacity to translocate the virions inside the cells.

Having come to this disappointing conclusion, we still believe that the phage vector we have designed may have utility as an imaging reporter agent. This concept does not rely on the gene delivery by this phage, and instead is based entirely on the specific recognition of the PSMA-expressing cells by this phage. This concept may be realized by engrafting the imaging compounds (radioactive or fluorescent) onto the surface of the PSMA-targeted phage particle. The amino terminus of the major component of the phage, pVIII protein, could be used for this purpose. Of note, it has been reported that short inserts, 6-8 aa residues can be incorporated into this locale with no deleterious consequences to the phage capsid assembly (7,8). For instance, pVIII could be genetically modified to contain a universal acceptor group such as an unpaired cysteine residue, which would then be used to conjugate the phage with a reporter (radioisotope, fluorescent protein, or Quantun dot). Alternatively, it could be modified by genetic incorporation of the *LUMIO* peptide (Invitrogen, Carlsbad, CA), which could then be loaded with a fluorescent reporter group. Since the capsid of a filamentous phage contains 2700 copies of pVIII protein, the reporter signal generated by a virion incorporating such modified pVIII should be enormous, thus justifying its use for tumor imaging.

Whereas the outcomes of our effort to make a PSMA-targeted Ad vector are undoubtedly disappointing, other researchers who tried to accomplish this goal failed, too. In this regard, recent work by Dr Rodriguez at John Hopkins, who is also working on PSMA-targeted Ad vector, resulted in the identification of peptides that apparently had some affinity for PSMA (9). However, subsequent effort by that group to configure these peptides into an Ad virion did not yield a desired vector (personal communication). Not only does PSMA remain an elusive target for peptide-based vector

development strategies, it appears that the whole concept of peptide-based targeting of Ad is rather unproductive, as very few successful targeting efforts have been reported. It would be fair to say that this approach does not work much more often than it does.

This is not to say that the vector that we originally proposed cannot be designed. Rather, it should be developed using alternative strategies. In this regard, our most recent work with new class of antibody mimicks, the so-called affibodies, has shown that these artificial proteins can be used to direct Ad vectors to desired tumor-specific targets. In particular, we have been able to use the recently developed affibodies with specificity to Her2 (10, 11) to target Ad vector to and kill the Her2-expressing breast cancer cells (unpublished work sponsored by the Susan G. Komen Breast Cancer Foundation). We are in the process of establishing formal collaboration with the Affibody AB (Bromma, Sweden) to initiate a broad effort to test other affibody ligands developed by this company. Should this work prove that the affibody ligands are universally suited for Ad tropism modification, we will apply this new strategy to develop a PSMA-specific Ad vector.

REFERENCES

1. Krasnykh, V. unpublished data.
2. Rasmussen, U. B., V. Schreiber, H. Schultz, F. Mischler, and K. Schughart. 2002. Tumor cell-targeting by phage-displayed peptides. Cancer Gene Ther 9:606-12.
3. Giordano, R. J., M. Cardo-Vila, J. Lahdenranta, R. Pasqualini, and W. Arap. 2001. Biopanning and rapid analysis of selective interactive ligands. Nat Med 7:1249-53.
4. Binz, H. K., P. Amstutz, and A. Pluckthun. 2005. Engineering novel binding proteins from nonimmunoglobulin domains. Nat Biotechnol 23:1257-68.
5. Magnusson, M. K., S. S. Hong, P. Henning, P. Boulanger, and L. Lindholm. 2002. Genetic retargeting of adenovirus vectors: functionality of targeting ligands and their influence on virus viability. J Gene Med 4:356-70.
6. Hajitou A, Trepel A, Lilley CE, Soghomonyan S, Alauddin M, Marin III FC, Restel BH, Ozawa MG, Moya CA, Rangel R, Sun Y, Zaoui K, Schmidt M, Von Kalle C, Weitzman MD, Gelovani JG, Pasqualini R and Arap W. 2006. A hybrid vector for ligand directed tumor targeting and imaging. Cell 125(2):385-98.
7. Iannolo G, Minenkova O, Gonfloni S, Castagnoli L, and Cesareni G. Construction, exploitation and evolution of a new peptide library displayed at high density by fusion to the major coat protein of filamentous phage. Biol Chem. (1997) 378(6):517-21.
8. Malik P, Terry TD, Gowda LR, Langara A, Petukhov SA, Symmons MF, Welsh LC, Marvin DA, and Perham RN. Role of capsid structure and membrane protein processing in determining the size and copy number of peptides displayed on the major coat protein of filamentous bacteriophage. J Mol Biol (1996) 260 (1) 9-21.
9. Lupold, SE, and Rodriguez R. Disulfide-constrained peptides that bind to the extracellular portion of the prostate-specific membrane antigen. 2004. Mol Cancer Ther. (5):597-603.

10. Orlova, A., M. Magnusson, T. L. Eriksson, M. Nilsson, B. Larsson, I. Hoiden-Guthenberg, C. Widstrom, J. Carlsson, V. Tolmachev, S. Stahl, and F. Y. Nilsson. 2006. Tumor imaging using a picomolar affinity HER2 binding affibody molecule. Cancer Res 66:4339-48.
11. Wikman, M., A. C. Steffen, E. Gunneriusson, V. Tolmachev, G. P. Adams, J. Carlsson, and S. Stahl. 2004. Selection and characterization of HER2/neu-binding affibody ligands. Protein Eng Des Sel 17:455-62.

APPENDICES

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